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Helicobacter pylori CagA Phosphorylation-Independent Function in Epithelial Proliferation and Inflammation

Masato Suzuki,[1] Hitomi Mimuro,[1] Kotaro Kiga,[1] Makoto Fukumatsu,[1] Nozomi Ishijima,[1] Hanako Morikawa,[1] Shigenori Nagai,[3] Shigeo Koyasu,[3] Robert H. Gilman,[4] Dangeruta Kersulyte,[5] Douglas E. Berg,[5] and Chihiro Sasakawa[1,2,6,*]

[1]Department of Microbiology and Immunology
[2]Department of Infectious Disease Control, International Research Center for Infectious Diseases
The Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan
[3]Department of Microbiology and Immunology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan
[4]Department of Microbiology, Facultad de Medecina, Universidad Peruana Cayetano Heredia, Lima 31, Peru
[5]Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, MO 63110, USA
[6]CREST, Japan Science and Technology Agency, Kawaguchi 332-0012, Japan
*Correspondence: sasakawa@ims.u-tokyo.ac.jp
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SUMMARY

CagA, a major virulence factor of *Helicobacter pylori* (Hp), is delivered into gastric epithelial cells and exists in phosphorylated and nonphosphorylated forms. The biological activity of the phosphorylated form is well established; however, function(s) of the nonphosphorylated form remain elusive. Here, we report that a conserved motif in the C-terminal region of CagA, which is distinct from the EPIYA motifs used for phosphorylation and which we designate CRPIA (*c*onserved *r*epeat responsible for *p*hosphorylation-*i*ndependent *a*ctivity), plays pivotal roles in Hp pathogenesis. The CRPIA motif in nonphosphorylated CagA was involved in interacting with activated Met, the hepatocyte growth factor receptor, leading to the sustained activation of phosphatidylinositol 3-kinase/Akt signaling in response to Hp infection. This in turn led to the activation of β-catenin and NF-κB signaling, which promote proliferation and inflammation, respectively. Thus, nonphosphorylated CagA activity contributes to the epithelial proliferative and proinflammatory responses associated with development of chronic gastritis and gastric cancer.

INTRODUCTION

Helicobacter pylori (Hp) chronically infects the stomachs of at least half the world's population, and numerous studies have indicated that persistent carriage of this bacterium increases the risk of several gastric diseases, including gastric adenocarcinoma (Montecucco and Rappuoli, 2001; Suerbaum and Michetti, 2002; Vogelmann and Amieva, 2007). CagA, a major Hp virulence factor that is produced by most strains of this species, is secreted via the type IV secretion system (T4SS) into gastric epithelial cells, where it plays a pivotal role in the etiology of Hp-associated gastric diseases (Backert and Meyer, 2006; Blaser et al., 1995; Ogura et al., 2000; Vogelmann and Amieva, 2007). Once within these target cells, some CagA molecules are tyrosine-phosphorylated by the Src/Abl kinase within unique EPIYA motifs (Poppe et al., 2007; Selbach et al., 2002; Stein et al., 2002; Tammer et al., 2007), while other CagA molecules remain unphosphorylated or become dephosphorylated (Selbach et al., 2003; Tsutsumi et al., 2003). The effects of CagA phosphorylation have been studied with particular intensity. Phosphorylated CagA causes dysregulation of epithelial structure and integrity through its effect on host cell signaling, such as the extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) pathway, by interacting with SHP-2, Grb2, Crk/Crk-L, Csk, Met, and ZO-1 (Amieva et al., 2003; Churin et al., 2003; Higashi et al., 2002; Mimuro et al., 2002; Suzuki et al., 2005; Tsutsumi et al., 2003). An important consequence of Hp infection, leading to gastric illnesses, is disruption of the adherens junctions (AJs) of the polarized gastric epithelial cells, resulting in nuclear localization of β-catenin (El-Etr et al., 2004; Suzuki et al., 2005), since β-catenin, a component of AJs, acts as a coactivator of Tcf/Lef (T cell factor/lymphoid enhancer factor) transcriptional factors in the nucleus (Clevers, 2006; Conacci-Sorrell et al., 2002). In epithelial cells with insufficient Wnt signaling, the GSK3β kinase component of the Axin/APC/GSK3β complex tightly regulates the free cytosolic pools of β-catenin; GSK3β kinase constitutively phosphorylates β-catenin and induces its degradation via the ubiquitin-proteasome system. Upon Wnt stimulation, β-catenin is stabilized by the inhibition of Axin/APC/GSK3β activity and is translocated into the nucleus. In turn, accumulation of β-catenin within the nucleus induces the expression of cancer-associated genes, such as Cyclin D1 and *c-myc* (Clevers, 2006; Conacci-Sorrell et al., 2002). Recent studies have indicated that CagA induces Tcf/β-catenin transcriptional activation, as does Wnt, though the mechanism is still unclear. Intriguingly, this activity does not require phosphorylation of the EPIYA motifs of CagA (Franco et al., 2005; Murata-Kamiya et al., 2007). Indeed, CagA can elicit diverse cell responses regardless of its phosphorylation state, including transcriptional activation of the serum response element (SRE), serum response

factor (SRF), nuclear factor-κB (NF-κB), and nuclear factor of activated T cells (NFAT) (Brandt et al., 2005; El-Etr et al., 2004; Hirata et al., 2002; Yokoyama et al., 2005). This strongly indicates that nonphosphorylated CagA also contributes to the development of the Hp-associated gastric illnesses, including gastric cancer. Nevertheless, the impact of phosphorylation-independent CagA functions in Hp-associated gastric carcinogenesis is still poorly understood. Here, we identify a motif in CagA that is distinct from the EPIYA motifs used for phosphorylation and that is essential for the phosphorylation-independent actions of CagA on target mammalian cells.

RESULTS

Nonphosphorylated CagA Activates Free β-Catenin via the PI3K/Akt Pathway

To investigate how Hp activates β-catenin during gastric epithelial cell infection, we created E-cadherin-expressing AGS (EAGS) cells by introducing the *CDH1* gene into the AGS gastric cell line, since the cells do not form functional AJs due to lack of E-cadherin expression, though the cells are highly susceptive to Hp infection (Suzuki et al., 2005) (Figure S1). When the EAGS cells were infected with Hp, we observed the efficient release of β-catenin from the AJ pools and its translocation into the nucleus (Figure 1A). Infection of EAGS with wild-type Hp, but not with Δ*cagA* (a *cagA*-deleted mutant), Δ*virD4* (a CagA secretion mutant), or Δ*virB7* (a T4SS-deficient mutant) elicited cell-cell dissociation, which was accompanied by Tcf/Lef transcriptional activation (Figures 1B and S2A). However, when the original AGS, KATOIII, or 293T cells (none of which have functional AJs) were infected with Hp, Tcf/Lef activation also occurred in a CagA-dependent manner (Figures 1B and S3). Furthermore, AJ disruption using an E-cadherin-neutralizing antibody was insufficient to induce the nuclear localization of β-catenin (Figure S2B). Hence, we reasoned that both the breakdown of AJs and the activation of free cytoplasmic β-catenin were required to activate Tcf/Lef-driven transcription by CagA during Hp infection.

To assess which cellular signaling pathways were involved in CagA-mediated β-catenin activation, we tested the effects of several pharmacological inhibitors on Tcf/Lef transcriptional activation during Hp infection. We found that LY294002 (a PI3K inhibitor), SB203580 (a p38 MAPK inhibitor), genistein (a tyrosine kinases inhibitor), and MG132 (a proteasome inhibitor) significantly blocked activation (Figure S4). We thus focused on the PI3K function on Hp-mediated β-catenin activation, since the p38 MAPK and 26S proteasome have been well known to generally regulate β-catenin signaling via its degradation (Clevers, 2006; Conacci-Sorrell et al., 2002; Edlund et al., 2005; Thornton et al., 2008). Wild-type Hp induced Akt kinase activation with phosphorylation on Ser-473 and Thr-308, and the Akt activation was completely abrogated by LY294002 treatment, suggesting that the PI3K/Akt signaling pathway was involved in CagA-mediated β-catenin activation (Figures 1C and 1D). In line with this, the Src/Abl kinase inhibitor PP2, which blocked CagA EPIYA motif phosphorylation, reduced the level of Akt activity but had no effect on the CagA-dependent increase in Akt activity (Figure 1D), suggesting that phosphorylation of CagA was dispensable for PI3K/Akt activation. The spatial distribution of the pleckstrin homology domain of Akt1 (PH-Akt) has been used as a good tracer for phosphatidylinositol-3,4,5-trisphosphate (PI[3,4,5]P_3), a major lipid product of PI3K (Pendaries et al., 2006). Thus, we infected AGS cells expressing PH-Akt-GFP (PH-Akt fusion with GFP) with bacteria and visualized the spatial distribution of PH-Akt. The results showed that the PH-Akt signals were accumulated around wild-type Hp attachment sites but not around Δ*cagA* Hp sites (Figure 1E). These results further suggested that the ability of CagA to activate the PI3K/Akt pathway was pivotal for stimulating Tcf/β-catenin transcriptional activation during Hp infection.

Class IA PI3K, which is involved in oncogenesis, is a heterodimer consisting of a p85 regulatory subunit and a p110 catalytic subunit (Koyasu, 2003; Vivanco and Sawyers, 2002). In Hp-infected AGS cells, CagA colocalized exactly with p85 (Figure 2A) and bound preferentially to its N-terminal SH2 domain in an EPIYA motif phosphorylation-dependent manner (Figures 2B and S5). Nevertheless, PR-CagA, which possesses Tyr/Phe substitutions in its EPIYA motifs that make it resistant to phosphorylation (Suzuki et al., 2005), was incapable of interacting with p85 but was still able to efficiently induce PI3K/Akt and PI3K/Akt-dependent β-catenin activation (Figures 2B and 2C). Moreover, native CagA and PR-CagA, but not ΔPY-CagA (a CagA mutant lacking the PY-region that contains the EPIYA motifs [Suzuki et al., 2005]), induced the transcriptional activation of Tcf/β-catenin and NF-κB (Figure 2D). This activation was blocked significantly by K179M-Akt (an Akt kinase-dead mutant) or phosphatase and tensin homolog (PTEN), which dephosphorylates PI(3,4,5)P_3 and functionally antagonizes PI3K/Akt signaling (Koyasu, 2003; Vivanco and Sawyers, 2002) (Figure 2E). In agreement with the above results, knockdown of a p85 subunit or a p110 subunit of PI3K or Akt also significantly reduced CagA-mediated β-catenin activation, though knockdown of SHP-2, Grb2, or Crk (all of which are known as CagA targets [Higashi et al., 2002; Mimuro et al., 2002; Suzuki et al., 2005]) hardly interfered with the CagA activity at all (Figures S8A–S8C and S8F). Furthermore, GSK3β, which is a downstream target of PI3K/Akt (Koyasu, 2003; Vivanco and Sawyers, 2002), was involved in both CagA-mediated β-catenin and NF-κB activation (Figure S6). Therefore, we hypothesized that the nonphosphorylated status of an uncharacterized CagA domain or domains, distinct from the well-known EPIYA motif, plays a pivotal role or roles in inducing the activation of PI3K/Akt signaling.

CagA Sequences Involved in PI3K/Akt Activation

To identify the putative CagA sequences involved in PI3K/Akt activation, we analyzed the amino acid sequences of the C-terminal PY regions of CagA from several clinical Hp isolates (Figure 3A). We found that there were repeat motifs that were highly conserved among CagA proteins; in European CagA, this sequence was FPLKRHDKVDDLSKVG, named CRPIA (*c*onserved *r*epeat responsible for *p*hosphorylation-*i*ndependent *a*ctivity) motif, which had also been named the CagA multimerization (CM) motif and found to bind to Par1b (Saadat et al., 2007) (Figure 3A). The CagA sequence of reference strain 26695 had two CRPIA/CM motifs, hereafter to be called CRPIA motif (positions 948–963 [CRPIA1] and 982–997 [CRPIA2]). Based on this sequence, we created a series of deletion mutants that had

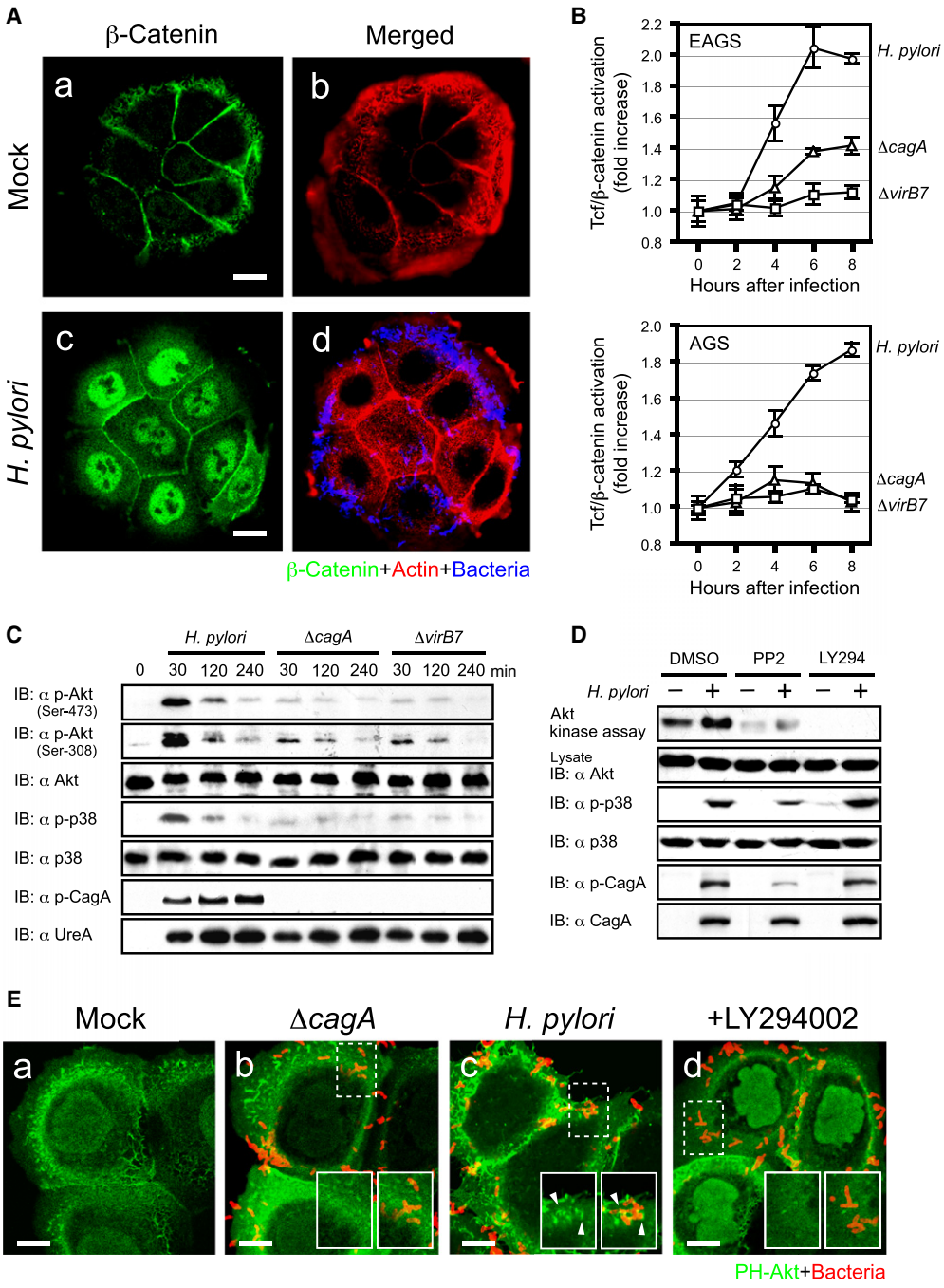

Figure 1. CagA Activates PI3K/Akt Signaling, Leading to β-Catenin Activation

(A) Immunostains of EAGS cells infected for 6 hr with Hp strain NCTC11637. Bar, 20 μm.

(B) Luciferase assays of EAGS and AGS cells transfected with the TOPflash luciferase reporter and infected with the indicated Hp strains.

(C) Immunoblots (IB) of AGS cells infected with the indicated Hp strains.

(D) Akt kinase assays and immunoblots of AGS cells treated with inhibitors and infected for 2 hr with Hp. (E) Immunostains of AGS cells transfected with PH-Akt-GFP and infected for 2 hr with Hp with or without inhibitors. The arrowheads show colocalization of Hp and PH-Akt. Bar, 10 μm.

truncated PY-regions (Figure 3B). Importantly, ΔPY2-CagA, which completely lacks EPIYA motifs (but has one CRPIA motif), retained sufficient activity to stimulate β-catenin and NF-κB activation (Figure 3D), although its stability was little different from that of native CagA (Figure 3C). Furthermore, ΔPY2-CagA could still partially induce cell scattering, a hallmark of CagA activity (Figures 3E and 3F). These results implied that the biological activity that was dependent on the CRPIA motif was substantially different from the activity that was dependent on the EPIYA motif and its phosphorylation.

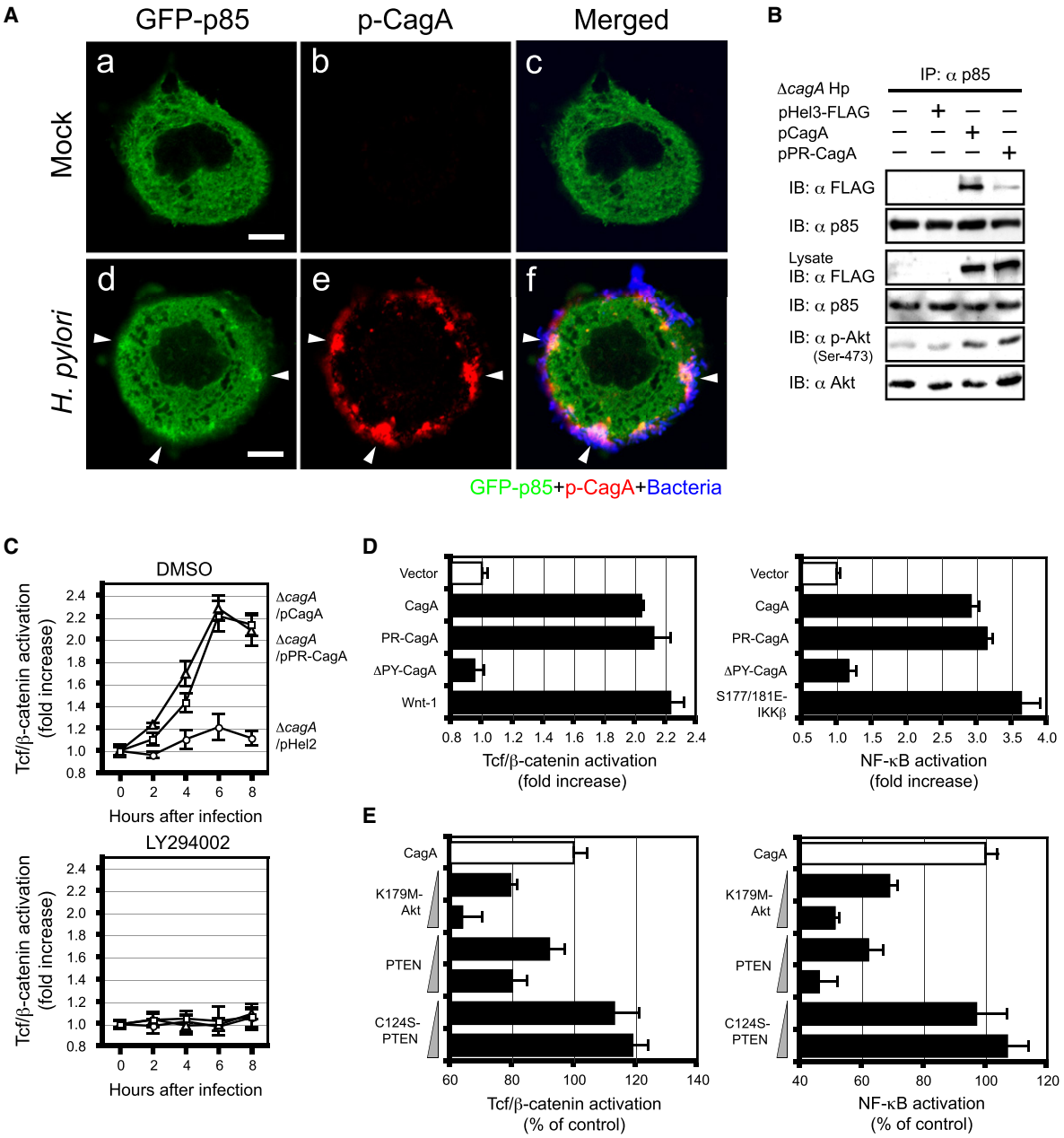

Figure 2. CagA Induces PI3K/Akt Activation Regardless of its Phosphorylation State

(A) Immunostains of AGS cells transfected with GFP-tagged p85 subunit of PI3K and infected for 2 hr with strain NCTC11637. The arrowheads show colocalization of intracellular CagA and GFP-p85. Bar, 20 μm.

(B) Immunoblots of lysates from AGS cells that were infected with Δ*cagA* Hp carrying the indicated pHel3-FLAG vectors for 2 hr, then subjected to immunoprecipitation (IP).

(C) Luciferase assays showing the transcriptional activity of Tcf/β-catenin from cells during infection, which were pretreated with inhibitors.

(D and E) Luciferase assays of 293T cells cotransfected with TOPflash or pNF-κB-Luc luciferase reporters along with CagA (250 ng), Wnt-1 (10 ng), or IKKβ (0.5 ng) (D) or CagA plus the indicated plasmids (125–250 ng) (E).

To further test this idea, we performed systematic alanine-scanning mutagenesis on positions 982–997, to identify the residues involved in the CRPIA motif activity. This series of mutants indicated that Phe-982, Leu-984, Lys-985, and Arg-986 were the critical residues for the biological activities of the CRPIA motif (Figure S7). In addition to their CRPIA motifs, CagA proteins have a conserved proline-rich motif in their PY-region (PEEP, residues at positions 913–916), which is likely a consensus PxxP sequence for recognition by an SH3 domain (Figure 3A). We thus constructed a series of full-length CagA point mutants such as PR (tyrosine-phosphorylation resistant), ΔPxxP (two Pro/Ala substitutions in the PxxP motif), ΔCRPIA1 (an

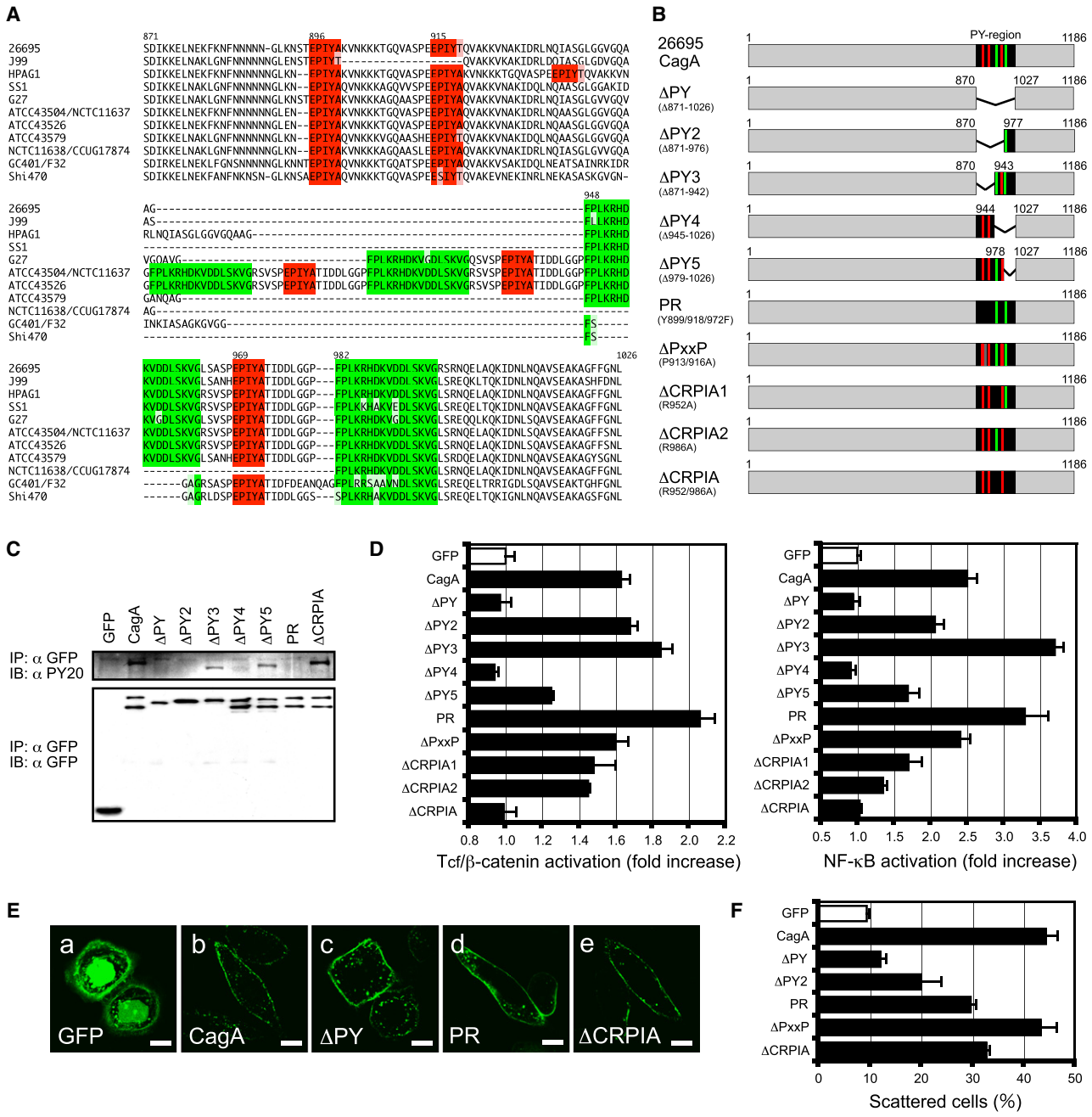

Figure 3. The CRPIA Motif Is Required for the EPIYA Motif-Independent Activity of CagA

(A) Multiple sequence alignment of the PY-regions of CagA from several Hp isolates.

(B) Schematic representations of 266695 CagA and a series of mutant derivatives. The EPIYA motifs (red) and CRPIA motifs (green) are highlighted.

(C) Immunoblots of 293T cells transfected with the indicated GFP plasmids that were subjected to immunoprecipitation.

(D) Luciferase assays of 293T cells cotransfected with TOPflash or pNF-κB-Luc and with GFP plasmids (250 ng).

(E) Fluorescence images of AGS cells transfected with GFP plasmids. Bar, 10 μm.

(F) The percentages of scattered cells in (E).

active-site Arg mutation in CRPIA1), ΔCRPIA2 (an active-site Arg mutation in CRPIA2), and ΔCRPIA (mutations in both CRPIA motifs) (Figure 3B). The activities of ΔPxxP-CagA, with respect to the stimulation of cell scattering and Tcf/β-catenin- and NF-κB-driven transcription, were almost the same as those of native CagA (Figures 3D–3F). On the other hand, the activities of ΔCRPIA were almost completely lost, and the activities of both ΔCRPIA1 and ΔCRPIA2 were partially decreased (Figure 3D).

To ensure the impact of the CRPIA motif on Hp infection, we used an Hp mutant that is defective in the nonphosphorylated CagA activity with mutations in its CRPIA motifs (ΔCRPIA-CagA in Figure 3B) and analyzed its CRPIA motif-associated

activities in vitro and in vivo. Although Hp expressing ΔCRPIA-CagA was still capable of injecting CagA into AGS cells to a similar extent as wild-type Hp (Figures 4A and 4C), the mutant strain could not elicit the activation of Akt kinase and downstream β-catenin and NF-κB late during AGS cell infection (Figures 4A and 4B). Moreover, 6 weeks after Hp infection of Mongolian gerbils, Hp expressing wild-type CagA colonized the gerbil stomachs more efficiently than Hp expressing ΔCRPIA-CagA ($p < 0.1$ in Mann-Whitney U test, Figure 4D). Immunohistochemical analyses of gastric antral sections from the infected gerbils revealed that stomachs infected with Hp expressing wild-type CagA, but not with Hp expressing ΔCRPIA-CagA, caused Akt hyperphosphorylation (Figure 4E). Thus, we concluded that the CRPIA motif plays a major role in stimulation of gastric PI3K/Akt signaling during Hp infection.

CagA Interaction with Activated Met

Unlike Hp expressing wild-type CagA, Hp expressing ΔCRPIA-CagA was unable to induce sustained activation of Met (Figure 4A). Importantly, immunostaining of AGS cells infected with Hp indicated that phosphorylated Met receptor tyrosine kinases (RTKs) were occasionally present beneath Hp expressing wild-type CagA, but not beneath Hp expressing ΔCRPIA-CagA (Figure 5A). As reported previously by Churin et al. (Churin et al., 2003), we investigated the involvement of the CagA CRPIA motif in Met activation using the Tpr-Met oncoprotein, a constitutively active Met mutant (Birchmeier et al., 2003; Peschard and Park, 2007). CagA and PR-CagA could interact with Tpr-Met, but not with K1110A-Tpr-Met (a kinase-dead mutant), while ΔCRPIA-CagA and ΔPY-CagA could not interact with either Met variant (Figure 5B). Furthermore, CagA could not interact with the Tpr-Met mutants that had Tyr/Phe substitutions in the multiple cytosolic docking site residues that are important for interactions with downstream signaling molecules (data not shown). Upon treatment with K252a (a Met RTK inhibitor [Morotti et al., 2002]) or knockdown of Met expression, the activation of Akt kinase and β-catenin during Hp infection of AGS cells was almost completely abrogated (Figures 5C and 5D and S8H). In addition, knockdown of Gab1, which is a critical adaptor protein for modulating Met signaling (Birchmeier et al., 2003; Peschard and Park, 2007), also prevented Hp-induced β-catenin activation (Figure S8H). Indeed, the activation of Met signaling resulted in the transcriptional activation of Tcf/β-catenin, NF-κB, SRE, SRF, NFAT, and activating protein-1 (AP-1), all of which are also responsible for CagA CRPIA motif-mediated signaling (Figures 5D–5I). In contrast, CagA did not affect the transcription of a control thymidine kinase minimal promoter (data not shown). Thus, the CagA interaction with Met via the CRPIA motif is likely to activate the variable signaling pathways downstream of Met.

Nonphosphorylated CagA Is Involved in the Proinflammatory Response to Hp Infection

CagA is a key determinant for the induction of NF-κB activation during Hp infection (Brandt et al., 2005; Kim et al., 2006) (Figure S6B); microarray analysis showed that Hp infection of epithelial cells induces the production of various chemokines, including IL-8, and that much of this induction depends on a functional *cagA* gene (El-Etr et al., 2004; Guillemin et al., 2002). Importantly, we found that CagA-induced *IL-8* mRNA expression and protein production depended on the presence of the CRPIA motifs in a long-term Hp infection (up to 24 hr [Figures 6A and 6B]). The impact of the CRPIA motifs was also seen in gastric primary cells from PI3K p85α subunit-deficient mice. Infection with Hp expressing ΔCRPIA-CagA could not induce the production of MIP-2 (macrophage inflammatory protein-2) and RANTES (regulated on activation, normal T cell-expressed and -secreted) as efficiently as infection with Hp expressing wild-type CagA (Figure 6C). In addition, upon Hp infection, the phosphorylation of the GSK3β and IκB kinases (IKKs) was increased to levels higher than those in noninfected cells, in a PI3K-dependent manner (Figure S9A). Moreover, the overexpression of the kinase-dead mutants of these kinases or the knockdown of their endogenous kinases reduced CagA-mediated cell responses (Figures S8G and S9B). Therefore, the CRPIA motif of CagA mediated the activation of PI3K/Akt signaling and its downstream effectors and facilitated the induction of proinflammatory responses during Hp infection.

The function of β-catenin is not only involved in the regulation of cell proliferation, but also in the regulation of the NF-κB-mediated proinflammatory response (Conacci-Sorrell et al., 2002; Deng et al., 2002; Spiegelman et al., 2000). NCI-H28, a human mesothelioma cell line that does not express β-catenin (Maeda et al., 2004) (Figure S10A), could still efficiently activate NF-κB transcriptional activation in response to Hp infection (Figure S10B). This suggests that the pathway for activating NF-κB that involves CagA is independent of β-catenin activation.

To further establish the CRPIA motifs as virulence-associated sequences of CagA, we used synthetic peptides composed of 16 residues, derived from the CRPIA motif, and investigated their inhibitory effects on CagA-mediated cell responses. The results showed that the CRPIA motif-derived peptide (CRPIA-peptide) was able to block the binding of the Met-binding domain (MBD) of CagA (residues 977–1026) to Tpr-Met, but a peptide with a single amino acid substitution at Arg-986 (CRPIAmt-peptide) could not (Figures 6D and 6E). Moreover, introduction of the CRPIA-peptide, but not the CRPIAmt-peptide, into 293T cells efficiently blocked CagA-dependent NF-κB activation and *IL-8* expression. In contrast, CRPIA-peptide had no effect on TNF-α-mediated NF-κB or *IL-8* activation (Figure 6F), further confirming the above results and suggesting that the blocking peptide specificity interfered with the CRPIA motif in CagA-mediated cell responses.

DISCUSSION

CagA is found in two states after injection into gastric epithelial cells: some CagA molecules undergo tyrosine phosphorylation at EPIYA motifs, while others remain unphosphorylated or become dephosphorylated. With fully one-third of the epithelial cell genes' expressions altered by CagA after Hp infection, the changes observed were not affected by the phosphorylation state of CagA (El-Etr et al., 2004). Previous studies had indicated that phosphorylated CagA caused a downregulation of Src family kinases activities, via both Csk-dependent and -independent pathways, and that this decrease in kinases activities exerted a feedback effect that decreased levels of phosphorylated CagA late during Hp infection in vitro (Selbach et al., 2003; Tsutsumi et al., 2003). Here, we provided further

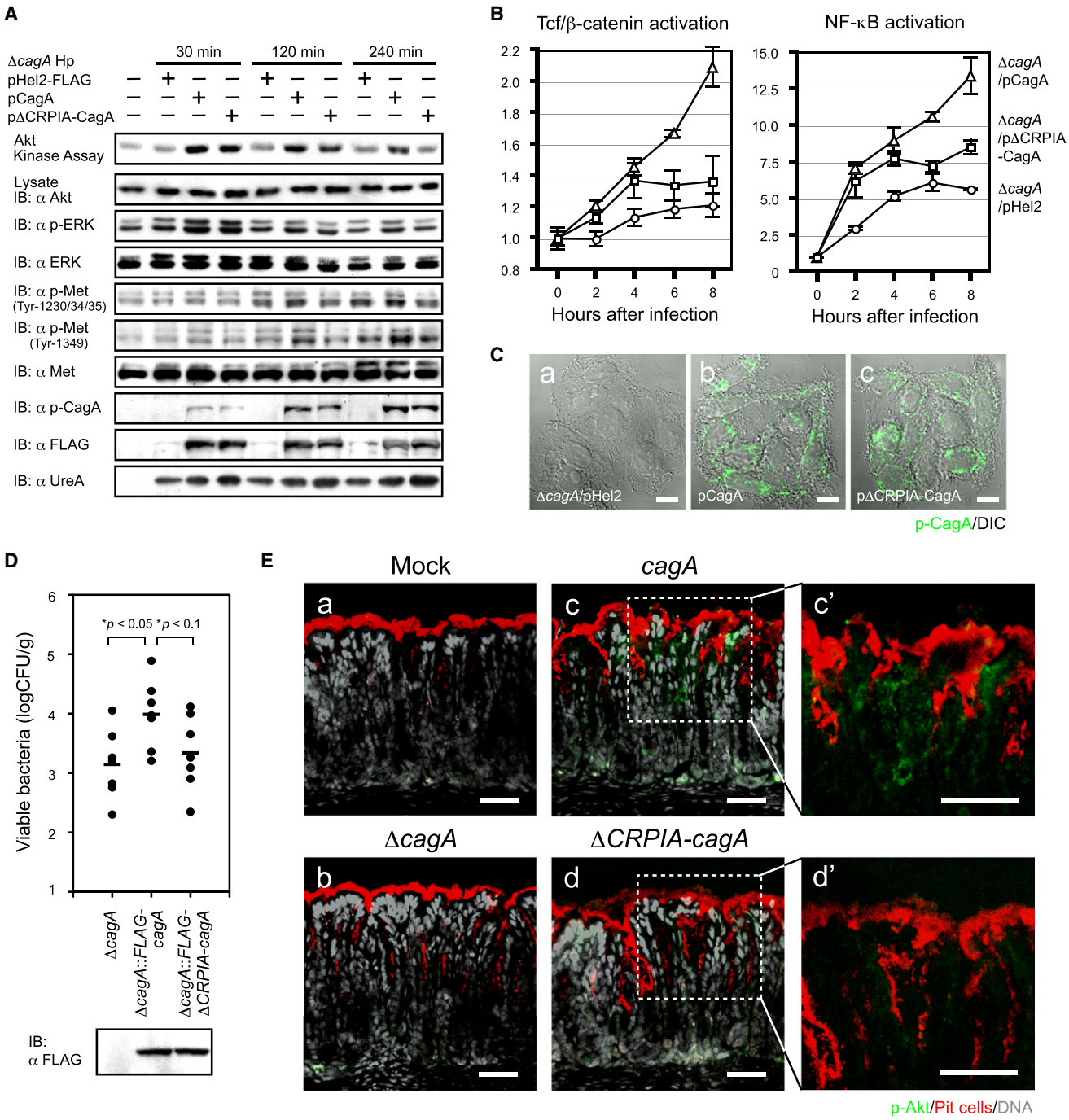

Figure 4. The CRPIA Motif Contributes to CagA-Mediated Sustained Host Cell Signaling

(A) Akt kinase assays and immunoblots of AGS cells infected with Δ*cagA* NCTC11637 carrying the indicated pHel2-FLAG vectors.

(B) Luciferase assays measuring the transcriptional activities of Tcf/β-catenin and NF-κB during infection.

(C) Immunofluorescence image showing intracellular localizations of CagA, observed using anti-p-CagA antibody 4 hr after infection. Differential interference contrast (DIC) images are also shown. Bar, 20 μm.

(D) Immunoblots showing FLAG-CagA expression (lower panel) and counts of viable bacteria (upper panel) from the stomachs of Mongolian gerbils that were infected for 6 weeks with the indicated ATCC43504 Hp strains. The data were statistically analyzed using a Mann-Whitney U test for unpaired groups.

(E) Immunostains of gastric antral sections from infected gerbils. Bar, 50 μm.

evidence that the nonphosphorylated form of CagA induces a variety of host cell proliferative and immune responses in gastric epithelial cells. We also showed that in Hp-infected gastric epithelial cells, the ability of CagA to interact with activated Met via its CRPIA motif was important for stimulating the downstream PI3K/Akt signaling and activating the pleiotropic

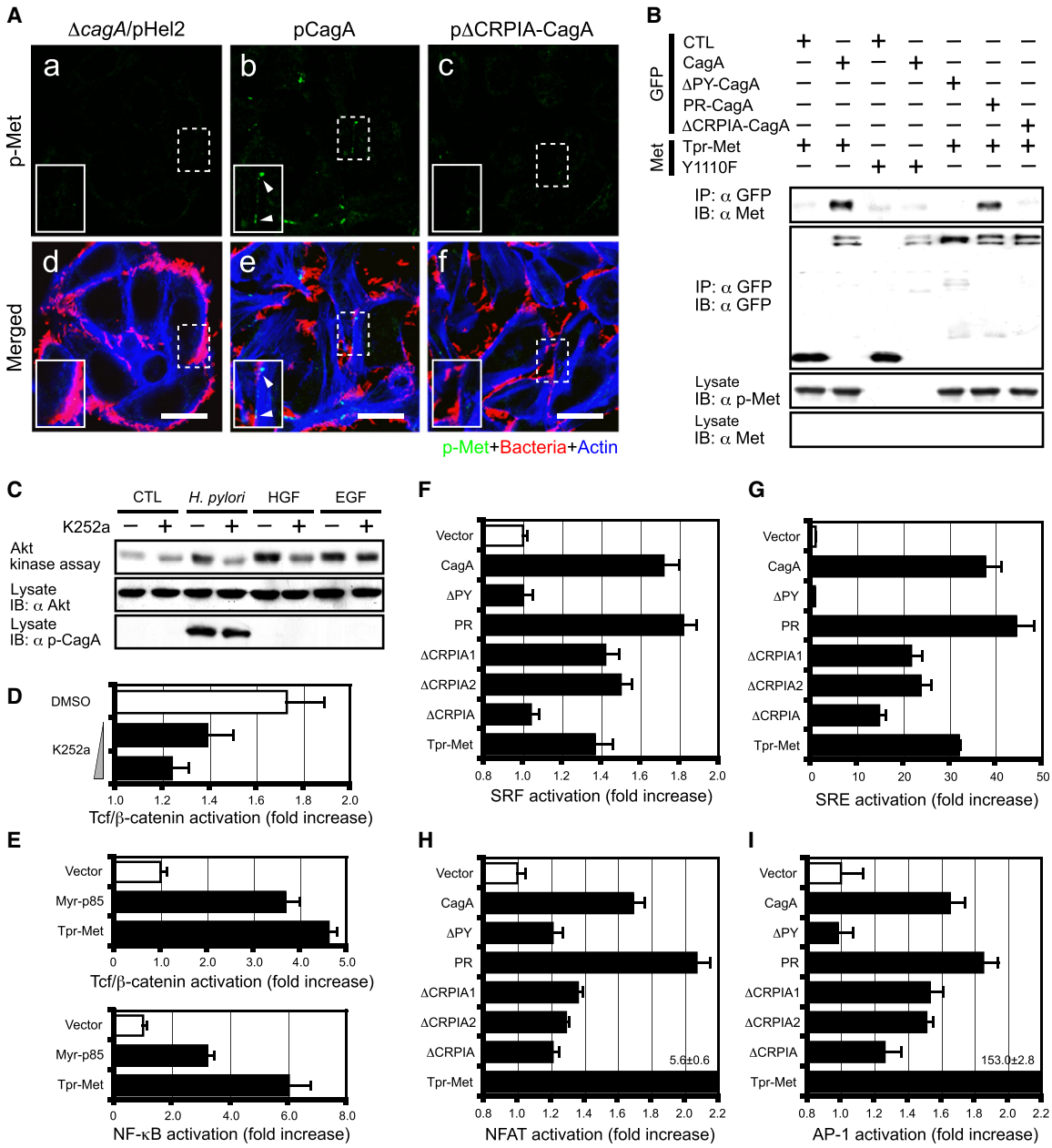

Figure 5. Targeting of CagA to Met Is Important for Activating Pleiotropic Signaling

(A) Immunostains of AGS cells infected for 2 hr with the indicated NCTC11637 derivatives. Arrowheads point to bacteria that are colocalized with p-Met. Bar, 20 μm.

(B) Immunoblots of 293T cells cotransfected with GFP and Tpr-Met plasmids that were subjected to immunoprecipitation.

(C) Akt kinase assays and immunoblots of AGS cells treated with DMSO or K252a (a Met RTK inhibitor) and stimulated with Hp, hepatocyte growth factor, or epidermal growth factor.

(D) Transcriptional activities of Tcf/β-catenin at 6 hr postinfection with K252a (100–200 ng/ml).

(E–I) Luciferase assays of 293T cells cotransfected with the indicated reporters and either GFP (250 ng), Myr-p85 (250 ng), or Tpr-Met plasmid (100 ng).

transcriptional responses, such as β-catenin and NF-κB. Others recently reported that in *cagA*-transfected MDCK cells, CagA protein bound to dimeric Par1b, a regulator of epithelial cell polarity, and multimerized at the cell plasma membrane (Saadat et al., 2007). We conclude that the CRPIA motif is important for sustaining cancer-associated transcriptional activation during chronic Hp infection.

It is worth noting that Gab1 also directly binds to activated Met via its unique Gab1 amino acid sequence, and that this binding is crucial for stimulating Gab1-mediated Met RTK signaling (Lock et al., 2003; Schaeper et al., 2000). Botham et al. recently reported that the loss of the *Drosophila* Gab1 homolog, which causes a defect in photoreceptor development in fruit flies, was rescued by the transgenic expression of CagA (Botham

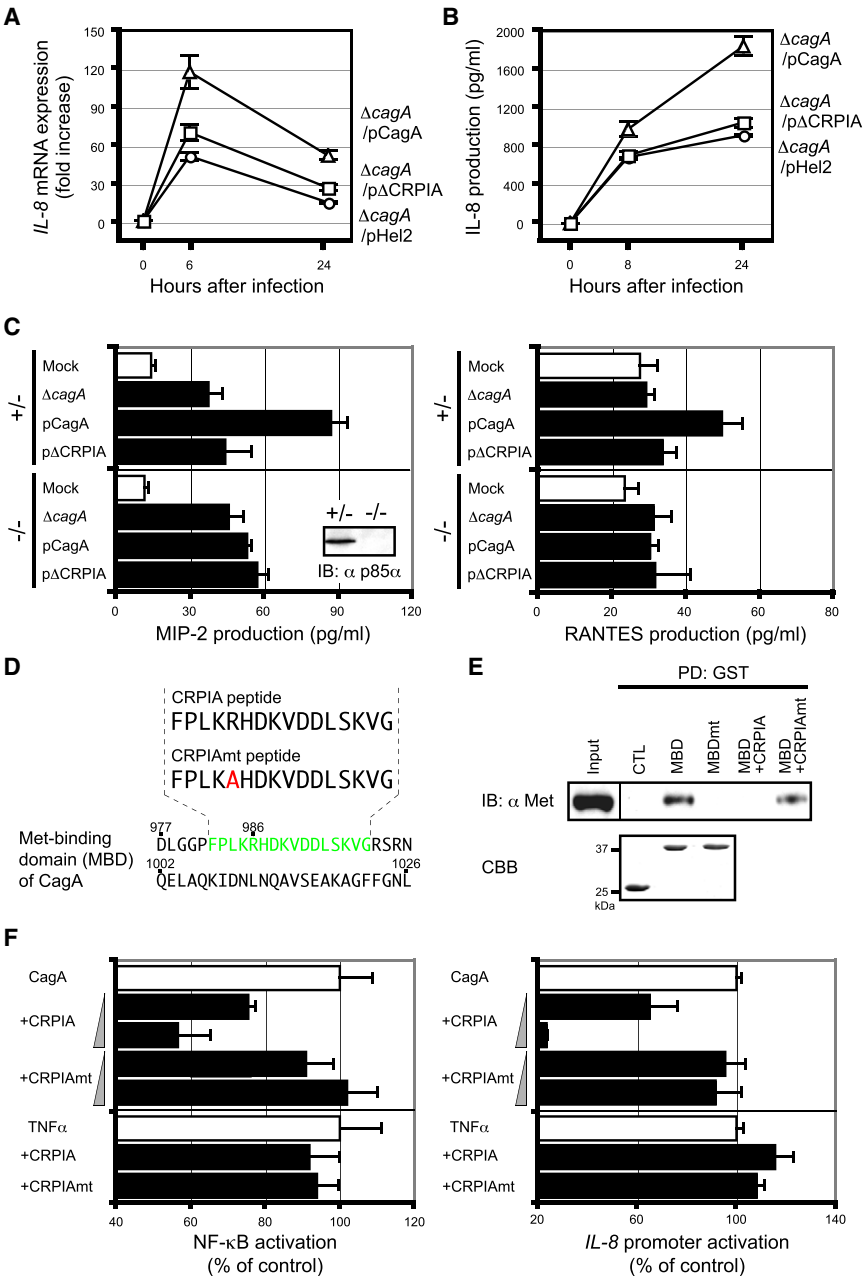

Figure 6. CagA Induces Proinflammatory Responses Depending on its CRPIA Motif

(A and B) AGS cells were infected with the indicated NCTC11637 derivatives. (A) shows RT-PCR assays for *IL-8* mRNA expression, and (B) shows ELISAs for detection of IL-8 from culture supernatants.

(C) ELISAs for the detection of MIP-2 and RANTES production by gastric primary cells from p85α-deficient mice (+/− and −/−) infected for 24 hr with the indicated Hp strains.

(D) Schematic representations of residues 977–1026 (MBD) of CagA and the sequences of the CRPIA motif-derivative peptides.

(E) Immunoblots of the pulled down proteins (PD) and lysates from AGS cells that were transfected with Tpr-Met and subjected to GST-pulldown assays using the indicated GST-CagA, with or without the peptides (200 μg/ml). CBB staining of the purified proteins is shown (lower panel).

(F) Luciferase assays of 293T cells cotransfected with the indicated reporters and the CRPIA motif-derivative peptides (2.5–10 μg/ml) and stimulated with CagA (250 ng) or with TNF-α (100 ng/ml).

infection, we had found that CagA upregulates MCL1, an antiapoptotic member of the Bcl2 family (Mimuro et al., 2007). In that study, CagA-mediated ERK/SRF signaling enhanced MCL1 production and promoted bacterial colonization in the gerbil stomachs, because it suppresses apoptosis and dampens the rapid turnover of the superficial pit epithelium. In the present study, we found that the CRPIA motif plays an important role in the upregulation of CagA-mediated activation of PI3K/Akt signaling in the in vivo Hp infection model (Figures 4D and 4E), and we confirmed that hyperphosphorylated Akt was predominantly expressed in the pit cells of Hp-infected gerbil stomachs. It is known that PI3K/Akt signaling influences cell survival via its indirect effects on at least two central cell death regulators, NF-κB and p53 et al., 2008). Although there is no amino acid sequence similarity between the CRPIA motif of CagA and the unique amino acid sequence of Gab1, we propose that CagA mimics the functional role of Gab1 in modulating the Met RTK downstream signaling pathways. If so, nonphosphorylated CagA would be likely to cause sustained Met/PI3K/Akt signaling late during Hp infection, an effect that would contribute to Hp-associated chronic gastric proliferative and proinflammatory responses (Figure S11). This is superimposed on numerous phosphorylation-dependent activities of CagA, which are most prominent at early time points (Figures 4A and 4B).

Given the above context, we wished to elucidate how CRPIA motif-mediated activity contributes to various cellular responses during Hp infection in vivo. Using a Mongolian gerbil model of Hp (Koyasu, 2003; Vivanco and Sawyers, 2002). Thus, we conclude that the nonphosphorylated CagA activity also plays a pivotal role in promoting the survival, multiplication, and dissemination of gastric epithelial cells, leading to Hp long-term colonization.

The β-catenin and NF-κB pathways intersect at several nodes: β-catenin can interfere with NF-κB activity by directly binding to NF-κB (Deng et al., 2002). Crosstalk also occurs through the Tcf/β-catenin-mediated transcription of the gene for E3 ubiquitin ligase β-TrCP, which recognizes both β-catenin and IκB (Spiegelman et al., 2000). IKKα directly phosphorylates and stabilizes cytoplasmic β-catenin, which is independent of the canonical Wnt/β-catenin pathway (Lamberti et al., 2001; Perkins, 2007). Furthermore, GSK3β, which forms a complex with Axin and APC, which is involved in proteasomal β-catenin degradation,

also plays a role in the regulation of NF-κB activation and target gene expression (Hoeflich et al., 2000). In the present study, we showed that an IκB supersuppressor had no effect on CagA-induced Tcf/β-catenin activation, and conversely, the loss of β-catenin did not affect CagA-induced NF-κB activation (Figures S9 and S10). We therefore assumed that CagA can activate NF-κB-mediated inflammatory signaling in a manner that is independent of β-catenin function. However, the expression of IL-8 in response to Hp infection, a key cause of Hp-induced inflammation, is regulated by Tcf/β-catenin in addition to NF-κB (Levy et al., 2002). Thus, the synergistic activity of Tcf/β-catenin and NF-κB may be important for inducing CagA-mediated IL-8 production. Matsumoto et al. recently reported that NF-κB activation caused the aberrant expression of activation-induced cytidine deaminase (AID), a key enzyme for antibody gene diversification, and excess AID in turn induced *p53* gene mutations during the Hp infection of gastric epithelial cells (Matsumoto et al., 2007). Here, we showed that CRPIA motif-mediated sustained PI3K/Akt activation causes NF-κB transcriptional activation during persistent Hp infection. Thus, it is tempting to speculate that the CRPIA motif activity of CagA also contributes to NF-κB-mediated *AID* gene expression and AID-mediated stomach tumorigenesis.

Finally, we note the implication of our findings for therapeutic strategies against Hp infection. Current therapies for Hp infection are antibiotics based and often compromised by antimicrobial resistance; few if any new anti-Hp antibiotics seem to be forthcoming (Gerrits et al., 2006). Here, we showed that synthetic CRPIA motif-derivative peptides greatly suppressed the proinflammatory response, a major pathogenic feature of Hp infection of the gastric epithelium and a major cause of gastric diseases, including gastric cancer. Therefore, we suggest that pharmacological inhibitors against the CRPIA motif-mediated activities of CagA are of potentially great value for avoiding the most debilitating consequences of chronic Hp infection.

EXPERIMENTAL PROCEDURES

Hp Infection in Cell Culture and Animals

The Hp strains NCTC11637, ATCC43504, ATCC43579, 26695, and B128 and their isogenic mutants, such as Δ*cagA*, Δ*virD4*, and Δ*virB7* have been described previously (Asahi et al., 2000; Mimuro et al., 2007, 2002; Suzuki et al., 2005). The Δ*cagA* NCTC11637 Hp strains harboring the pHel3-FLAG vectors have been described previously (Suzuki et al., 2005). The Δ*cagA* NCTC11637 Hp strains (harboring the pHel2-FLAG vectors) and the Δ*cagA* ATCC43504 Hp strains (complementing the *cagA* gene with chromosome) were constructed for this study. All Hp strains were cultured according to standard procedures (Suzuki et al., 2005). The AGS, EAGS, and 293T cells were maintained in Dulbecco's modified Eagle's medium (Sigma-Aldrich; St. Louis, MO) containing 10% fetal bovine serum (FBS). The BING, KATOIII, and NCI-H28 cells were maintained in RPMI-1640 (Sigma-Aldrich) containing 10% FBS. Cultured cells were infected with Hp at a multiplicity of infection (MOI) of 100. Hp infection of AGS cells or CagA plasmid transfection of the cells induced "cell scattering," which was also recognized by the "hummingbird" phenotype described previously (Suzuki et al., 2005). p85α-deficient Balb/c mice have been described previously (Suzuki et al., 1999), and the primary gastric epithelial cells were prepared as described previously (Mimuro et al., 2007). Hp infection of Mongolian gerbils was performed as described previously (Nagai et al., 2007). Briefly, 6-week-old male MGS/Sea Mongolian gerbils (CLEA Japan, Inc.; Tokyo, Japan) were intragastrically inoculated with an Hp culture containing 10^9 CFU, and their stomach sections were used for immunohistological analysis 6 weeks later. Animal experiments were conducted in accordance with the University of Tokyo guidelines for the care and use of laboratory animals and were approved by the ethics committee for animal experiments at the University of Tokyo.

Plasmid Construction

The Tcf/Lef luciferase reporter constructs TOPflash and FOPflash were obtained from Upstate Biotechnology; Lake Placid, NY. The NF-κB (pNF-κB-Luc), SRE, SRF, and AP-1 luciferase reporter constructs were obtained from Stratagene; La Jolla, CA . The NFAT luciferase reporter construct and the *Renilla* luciferase vector phRL-TK were obtained from Promega; Madison, WI. The human *IL-8* promoter (−1498/+44 fragment) was amplified from the genomic DNA of HeLa cells and cloned into pGL4.20 (Promega). The p85α subunit of PI3K and the *PTEN* cDNA were amplified by RT-PCR from the total RNA of Caco-2 cells and were cloned into pcDNA3.1 (Invitrogen; Carlsbad, CA). The Src myristoylation signal-tagged p85 (Myr-p85) was generated using site-directed mutagenesis. The Wnt-1, Akt1, and K179M-Akt expression vectors were obtained from Upstate Biotechnology, and the PH domain of Akt1 (residues 1–131) was subcloned into pEGFP-N1 (Clontech) to detect the spatial distribution of $PI(3,4,5)P_3$. The Tpr-Met expression vector was kindly provided by Dr. M. Park (McGill University; Montreal, Quebec, Canada), and the K1110A-Tpr-Met mutant was generated using site-directed mutagenesis. The *Escherichia coli*-Hp shuttle vectors, pHel2 and pHel3, were kindly provided by Dr. R. Haas (Ludwig Maximilians University; Munich, Germany). The N-terminal FLAG-tagged full-length, PR, and ΔPY derivatives of NCTC11637 CagA in pHel3 were described previously (Suzuki et al., 2005), and each CagA was subcloned into pCXN2 for the transfection of mammalian cells. The *cagA* gene from 26695 Hp was cloned into pEGFP-C1 (Clontech), and the deletion and point mutants of CagA, such as ΔPY (deletion of residues 871–1026), ΔPY2-CagA (deletion of residues 871–976), PR (Y889/918/972F), and ΔCRPIA (R952/986A), were generated using site-directed mutagenesis. Full-length, PR, and ΔCRPIA CagA were subcloned into pHel2 with an N-terminal FLAG-tag for the transcomplementation of CagA expression in Δ*cagA* Hp. The MBD of CagA (residues 977–1026) and the Arg-986 mutant were subcloned into pGEX6P1 (Amersham Biosciences; Buckinghamshre, England) to prepare the glutathione S-transferase (GST) fusion proteins.

GST-Pulldown Assay, Immunoprecipitation, Immunoblotting, and Immunostaining

GST-pulldown assays, immunoprecipitation, immunoblotting, and immunostaining using the appropriate purified proteins and antibodies (the venders of the antibodies and reagents are shown in Supplemental Data) were performed as described previously (Mimuro et al., 2007; Suzuki et al., 2005). Following immunostaining, the specimens were observed using a Confocal Laser-Scanning Microscope (LSM 510; Carl Zeiss), and the fluorescence images were analyzed using LSM 510 version 3.2 software (Carl Zeiss; Oberkochen, Germany). The Akt kinase assays were performed using the Akt Kinase Assay Kit (Cell Signaling Technology; Beverly, MA) according to the manufacturer's instructions.

DNA and Peptide Transfection

Transient transfections with the appropriate plasmids were carried out using FuGene6 (Roche; Basel, Switzerland), Lipofectamine 2000, or Lipofectamine LTX (Invitrogen). Transient transfections with the appropriate synthetic peptides were carried out using Profect P1 (Targeting Systems; Santee, CA) according to the manufacturer's instructions. The CagA CRPIA motif derivative-peptides (FPLKRHDKVDDLSKVG and FPLKAHDKVDDLSKVG) were synthesized, and their identities were verified by the Laboratory Center for Proteomics Research, Institute of Medical Science, the University of Tokyo (Tokyo, Japan).

Luciferase Assay

Luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. The levels of the firefly luciferase activities were measured and normalized to the activity of phRL-TK-derived *Renilla* luciferase. The results are expressed as the means ± SEM from triplicate experiments.

Real-Time PCR

Total RNA was reverse transcribed into cDNA with oligo (dT) primers, then amplified and quantified by detection of SYBR Green using a LightCycler DX400 (Roche). Relative mRNA expression was calculated using the expression of the human GAPDH gene as an endogenous reference standard. Primer sequences were selected with LightCycler Probe DesignSoftware 2.0 (Idaho Technology). The results are expressed as the means ± SEM from triplicate experiments.

ELISA

Enzyme-linked immunosorbent assays (ELISAs) for human IL-8 were performed using the Human IL-8 ELISA Kit (Pierce Biotechnology; Rockford, IL), and ELISAs for mouse MIP-2 and RANTES were performed using the Quantikine ELISA Kit (R&D Systems; Minneapolis, MN) according to the manufacturer's instructions. The results are expressed as the means ± SEM from triplicate experiments.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, and 11 figures and can be found online at http://www.cell.com/cellhostandmicrobe/supplemental/S1931-3128(08)00402-2.

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